Comparative studies on peptides representing the so-called tachykinin-like region of the Alzheimer A β peptide [A β (25–35)]

Omar M. A. EL-AGNAF*, G. Brent IRVINE*, Geraldine FITZPATRICK†, W. Kenneth GLASS† and David J. S. GUTHRIE*¹

*The Centre for Peptide and Protein Engineering, School of Biology and Biochemistry, The Queen's University of Belfast, 97 Lisburn Road, Belfast BT9 7BL, N. Ireland, U.K., and †Department of Chemistry, University College Dublin, Belfield, Dublin 4, Republic of Ireland

In an attempt to answer the question of whether or not the so-called tachykinin-like region of the Alzheimer β -amyloid protein [A β (25–35)] can act as a tachykinin, the sequences A β (25–35), A β (25–35)amide and their norleucine-35 and phenylalanine-31 analogues were synthesized. These peptides were examined with ligand binding studies, electron microscopy, CD and NMR. In all cases some differences were found between the A β (25–35)

analogue and the corresponding Phe³¹ peptide. In addition, in ligand displacement studies on tachykinin NK1 receptors, only the Phe³¹ analogue showed activity comparable to that of genuine tachykinins. We conclude that peptides based on A β (25–35) but with a Phe residue at position 31 do display properties typical of a tachykinin, but that peptides with Ile at this position do not.

INTRODUCTION

Alzheimer's disease is today the most common neurodegenerative disease of the elderly that causes dementia. A major characteristic is the occurrence in the brain of senile plaques. These are masses of insoluble, aggregated proteins whose main constituent is the β -amyloid protein (A β), a 39–43-residue peptide derived from the processing of a much larger protein called the amyloid precursor protein [1].

A β (and some fragments thereof) have been shown to possess properties consistent with their having a major role in the onset and progression of Alzheimer's disease. First, the peptides aggregate from buffer solutions to produce fibrils that resemble those found in natural plaques [2,3]. This aggregation is believed to involve individual molecules of A β adopting an extended structure and associating to form a β -sheet [3,4]. Further association in an ordered manner continues until the assembly exceeds the solubility limits and fibrils are deposited. Secondly, A β is known to affect neuronal behaviour both *in vivo* and *in vitro*. For example, A β is neurotoxic when injected into rat brain [5] or when administered to neurons growing in culture [6–8]. These properties seem to be linked in that several authors report that only amyloid peptides that are competent to form fibrils, or at least to adopt a β -sheet structure, display neurotoxicity. For example, freshly prepared solutions of A β alter on aging in such a way that as the degree of β -sheet content increases, the resultant conformers exhibit increased neurotoxicity [4,9,10].

Yankner and co-workers reported a significant advance in 1990 [6] with the demonstration that the fragment A β (25–35) retained the neurotoxic properties of the full molecule. They also drew attention to the sequence similarity between A β and the tachykinin family of neuropeptides, a widely distributed family of peptides whose activity resides in a characteristic C-terminal pentapeptide sequence Phe-Xaa-Gly-Leu-Met-NH₂. The Phe residue and the amide function are essential for receptor binding and for biological activity [11]. Some examples of tachykinins are compared with amyloid sequences in Table 1.

Yankner [6] reported that the presence of substance P and physalaemin effectively countered the effects of amyloid peptides on neurons in culture. Neurokinin B gave partial protection, whereas neurokinin A and eldoisin were without effect. Some

tachykinin antagonists such as Spantide, which were already known to be neurotoxic *in vivo* [12], also replicated the deleterious effects of amyloid peptides on neurons in culture. Yankner and co-workers therefore suggested that amyloid peptides might mediate their effects via tachykinin receptors. In response to this suggestion, numerous papers have appeared that investigated the interaction of amyloid peptides with tachykinin receptors isolated from a variety of sources, and also the effects of tachykinin agonists and antagonists on the activity of amyloid peptides (see references in [13]).

In general the results obtained have not borne out Yankner's hypothesis, the amyloid peptides tested showing at best only very weak receptor binding [14–16]. In particular, both A β (25–35) [14] and A β (25–35)amide [15] failed to displace labelled substance P from NK1 (substance P) receptors isolated from rat brain. In addition, functional assays have also failed to show an interaction of A β (25–35) with tachykinin receptors [17,18], although again some positive results have been reported [19]. More complex interactions have been proposed, for example the binding of both tachykinins and amyloid peptides to the serpin-enzyme complex receptor rather than to tachykinin receptors [20,21] and also the interaction of A β (1–40) with tachykinin receptors leading to inositol triphosphate production and an increase in intracellular [Ca²⁺] in the presence of glutamate, glycine and CaCl₂.

Table 1 Amino acid sequences of peptides discussed in the present paper

Amino acid codes in lower-case, e.g. arg, denote a D-amino acid.

Peptide	Structure
A β (25–35)	Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-OH
Substance P	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH ₂
Neurokinin A	His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH ₂
Neurokinin B	Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-Met-NH ₂
Physalaemin	pGlu-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu-Met-NH ₂
Eldoisin	pGlu-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met-NH ₂
A β [Phe ³¹](25–35)	Gly-Ser-Asn-Lys-Gly-Ala-Phe-Ile-Gly-Leu-Met-OH
A β [Phe ³¹ ,Nle ³⁵](25–35)amide	Gly-Ser-Asn-Lys-Gly-Ala-Phe-Ile-Gly-Leu-Nle-NH ₂
A β [Nle ³⁵](25–35)amide	Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Nle-NH ₂
Spantide	arg-Pro-Lys-Pro-Gln-Gln-trp-Phe-trp-Leu-Leu-NH ₂

Abbreviations used: A β , β -amyloid protein; Nle, norleucine; ROESY, rotating-frame Overhauser spectroscopy; TFE, trifluoroethanol.

¹ To whom correspondence should be addressed (e-mail d.guthrie@qub.ac.uk).

[22]. This latter result is consistent with recent studies reporting interactions between tachykinin receptors and excitatory amino acids, especially interactions with the *N*-methyl-D-aspartate receptor [23]. Finally, with the use of a different assay from that used by Yankner [6], Shearman [13] failed to reproduce Yankner's findings that substance P and some other tachykinins gave protection against the neurotoxic effects of A β (25–35).

We preferred to approach the problem from a different direction. If A β (25–35) really were a tachykinin, it would have a Phe residue at position 31 and also an amide function at the C-terminus. From Table 1 it can be seen that A β [Phe³¹](25–35)amide is identical with the tachykinin eledoisin in 7 out of 11 residues and might be expected to have many of the typical properties of a tachykinin. We therefore synthesized some analogues of A β [Phe³¹](25–35)amide and compared a range of their properties with those of the corresponding A β (25–35) analogues. We looked for similarities and differences between the properties of the A β (25–35) and A β [Phe³¹](25–35) sequences and for evidence that would show only the latter peptides to have properties typical of tachykinins.

EXPERIMENTAL

Peptide synthesis

The peptides A β (1–40), A β [Gly²¹](1–40) and A β [Gln²³](1–40) were a gift from Dr. D. Allsop. Other peptides were synthesized on Rink polystyrene resins by using fluorenyl-9-ylmethoxycarbonyl N-protection as described by El-Agnaf et al. [24]. The peptides were isolated either by precipitation from the cleavage solution by the addition of dry diethyl ether or by freeze-drying from 5% (v/v) aqueous acetonitrile containing 0.1% (v/v) trifluoroacetic acid [25]. The identity and purity of the peptides were assessed by amino acid analysis, reverse-phase HPLC, capillary electrophoresis and fast-atom bombardment or electrospray ionization MS. If purity was judged to be less than 95%, peptides were purified by reverse-phase HPLC on C₁₈ columns.

Receptor binding studies

Rat brain membranes were prepared by a slight modification of the method of Lee and Snyder [26]. Whole brains (except cerebella) were rapidly removed from adult rats and kept on ice. The brain tissue was homogenized in 10 vol. of ice-cold buffer A (50 mM Tris/HCl, pH 7.40, containing 120 mM NaCl and 5 mM KCl) with a hand-driven Teflon homogenizer (12 hand strokes of the pestle) to minimize frothing. The homogenate was centrifuged at 50 000 *g* for 10 min at 4 °C and the supernatant was discarded. The pellet was subjected to three washings by resuspension, once in 10 vol. of ice-cold buffer A and twice in 20 vol. of ice-cold buffer B (50 mM Tris/HCl, pH 7.40), after which the suspension was centrifuged at 4 °C and the supernatant was discarded. The final pellet was suspended in 20 vol. of buffer B for use. Binding assays were performed in Eppendorf tubes (1.5 ml) precoated with Aquasil.

Routinely 100 μ l of membrane suspension were incubated in 400 μ l (final volume) of buffer (50 mM Tris/HCl, pH 7.40, containing 0.02% BSA) containing a cocktail of protease inhibitors (0.2 mg/100 ml chymostatin, 0.4 mg/100 ml leupeptin, 4 mg/100 ml bacitracin, and 1 μ M phosphoramidon) and also MnCl₂ (3 mM). Incubations were performed for 20 min at 20 °C with ¹²⁵I-labelled Bolton and Hunter substance P (Amersham) in the presence or absence of unlabelled ligand or of an analogue over the concentration range 10^{–11}–10^{–4} M. At the end of the

incubation the tubes were centrifuged for 30 s at 10 000 *g* and the pellets were washed three times with ice-cold deionized water by consecutively dipping the tube carefully into three beakers (each containing approx. 250 ml of de-ionized water) and equally carefully pouring off the supernatant water. The radioactivity in the pellets was measured with a γ -counter (Nuclear Counterpriss NE 1600). Non-specific binding was found to be less than 29%. The results are presented as means $\pm$ S.E.M. When the magnitude of the error is of the same order as the symbol size, no error bars are visible.

Aggregation studies

Peptides (100–350 μ g) were weighed into a glass vial, dissolved in water at twice the desired final concentration and diluted with an equal volume of double-strength buffer (either 100 mM Tris/HCl, pH 7.4, or 2 $\times$ PBS, pH 7.4). All solutions also contained 0.025% sodium azide as anti-microbial agent. The solution was immediately transferred to a 1.5 ml Eppendorf tube and centrifuged at 17 500 *g*_{max} for 10 min. After 4 days any precipitate was collected by centrifugation for further study.

Electron microscopy

Electron micrographs were produced from pellets obtained by centrifugation at the end of the aggregation assay. The pellets were resuspended in water (100 μ l) by vortex-mixing and an aliquot (5 μ l) was placed on a carbon-coated Formvar grid and allowed to dry in air before being negatively stained with 2% (w/v) uranyl acetate for 2 min. Samples were then observed under a JEOL TEMCX 100 II electron microscope.

CD spectroscopy

CD spectra were recorded over the range 190–250 nm on a Jasco J720 spectropolarimeter. An aliquot of the peptide solution (approx. 100 μ l) was placed in a quartz cell (0.2 mm path length). All spectra were corrected by subtraction of the baseline of the appropriate solvent. Results are expressed as molar ellipticity, $[\theta]_M$, in units of deg $\cdot$ cm² $\cdot$ dmol^{–1}. Solutions of peptides in buffer or mixed organic/aqueous solvents were prepared by dissolving freeze-dried peptide in water and dilution of this solution by addition of an appropriate volume of buffer solution or the relevant organic solvent.

NMR spectra

¹H NMR spectra were recorded from peptides dissolved in [²H₆]DMSO (approx. 8 mM) on a Varian Unity NMR spectrometer operating at 500 MHz. Resonance assignments were made on the basis of phase-sensitive double-quantum-filtered COSY spectra, phase-sensitive NOESY spectra and rotating-frame Overhauser spectroscopy (ROESY) spectra. These two-dimensional spectra were acquired with 2K data points in the F2 dimension and 256 increments in the F1 dimension. Before application of the Fourier transform, the results were zero-filled to give a 2K $\times$ 2K data matrix and multiplied by a cosine function. All spectra were obtained with standard Varian procedures and processing was performed with the current Varian NMR software. NOESY spectra were acquired with mixing times of 200 and 400 ms, and ROESY spectra with mixing times of 200 and 700 ms.

Coupling constants (³*J*_{NH}) were obtained from high-resolution one-dimensional spectra. The temperature dependence of amide protons was also determined from one-dimensional spectra

recorded at 5 °C intervals from 25 to 50 °C. The variation of the chemical shifts of the amide protons with temperature was found to be linear. In some cases overlapping resonances obscured individual resonances but where four or more data points could be collected the temperature coefficient was determined by linear regression analysis (correlation coefficients at least 0.99).

RESULTS AND DISCUSSION

In seeking to compare the properties of A β (25–35) with those of a Phe³¹ analogue, a decision has to be made over the nature of the C-terminus. There is no known pathway by which a peptide with a C-terminal methionine amide could be formed *in vivo* from either A β or the amyloid precursor protein, because the residue in A β after Met³⁵ is Val, not Gly [27]. However, a true tachykinin must have an C-terminal amide for activity and peptides with a tachykinin sequence but with a C-terminal acid function do not bind to tachykinin receptors [11]. To permit the fullest possible comparisons, we have synthesized the peptides A β (25–35) and A β [Phe³¹](25–35) with both C-terminal acid and C-terminal amide functions and also with Met³⁵ replaced by norleucine (Nle).

Differences between the sequences were found even during the synthesis of the peptides. These have already been described [24] and will only be summarized here. We chose initially to synthesize peptides with Met³⁵ replaced by Nle. This is a reasonably common modification used in tachykinin studies (see Table 1 and [28]). It generally causes a modest decrease in biological activity compared with the methionine peptide [11] but removes the possibility of the properties of the peptide altering as the Met³⁵ undergoes oxidation, especially during prolonged incubations in solution.

As expected, the synthesis of the peptide A β [Phe³¹,Nle³⁵](25–35)amide proceeded smoothly to completion under standard conditions (dimethylformamide as solvent and 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate as coupling agent). Unexpectedly, under the same conditions the synthesis of A β [Nle³⁵](25–35)amide came to a stop after the addition of Ala³⁰ and could not be continued by multiple couplings of the next residue, even with different coupling methods. An attempt to synthesize A β [Nle³⁵](25–35) similarly failed at the same position. It was found possible to complete the syntheses either by using for the coupling steps a solvent system (dimethylformamide/DMSO/*N*-methylpyrrolid-2-one), which more effectively solvates the growing peptide chains, or by coupling Ala³⁰ as an *N*-hydroxymethoxybenzyl derivative, which prevents interchain or intrachain associations. Thus we concluded that A β [Nle³⁵](25–35)amide and A β [Nle³⁵](25–35) were more prone to form interchain or intrachain associations than was A β [Phe³¹,Nle³⁵](25–35)amide or indeed any of the other tachykinin analogues that we had synthesized previously [28,29].

After synthesis the peptides had different solubility properties. When isolated by precipitation from trifluoroacetic acid solution by the addition of dry diethyl ether, the Phe³¹ peptides dissolved easily in water or buffer, whereas all the A β (25–35) analogues were insoluble in buffer and only sparingly soluble in water [25]. When isolated by freeze-drying from 5% (v/v) aqueous acetonitrile containing 0.1% (v/v) trifluoroacetic acid, all the peptides dissolved readily in water; peptides so treated were used in all further studies.

As mentioned above, it has previously been reported that both A β (25–35) [14] and A β (25–35)amide [15] at concentrations up to 10 μ M fail to displace radiolabelled substance P analogues from rat brain membrane preparations containing NK1 receptors. The former result is not surprising because even the substance P

Figure 1 Displacement of ¹²⁵I-labelled Bolton and Hunter substance P from rat brain membrane preparations by substance P (□), neurokinin A (◇), neurokinin B (○), A β [Phe³¹,Nle³⁵](25–35)amide (△) and A β [Nle³⁵](25–35)amide (⊠)

Figure 2 Displacement of ¹²⁵I-labelled Bolton and Hunter substance P from rat brain membrane preparations by substance P (□), A β [Nle³⁵](25–35)amide (⊠), A β [Nle³⁵](23–35)amide (○), A β [Nle³⁵](21–35)amide (△) and A β [Nle³⁵](19–35)amide (◇)

sequence with a C-terminal carboxy group has been shown not to bind to tachykinin receptors [11]. We compared the ability of A β [Nle³⁵](25–35)amide and A β [Phe³¹,Nle³⁵](25–35)amide to bind to NK1 receptors present in rat brain membranes by using the displacement of ¹²⁵I-labelled Bolton and Hunter substance P (Figure 1). To examine the specificity of the radioligand binding to the rat brain tachykinin receptors, the ability of the three mammalian tachykinins to displace this ligand from rat brain membranes was studied. The three tachykinins were found to displace the label with affinities in the order substance P > neurokinin A > neurokinin B, indicating that we were dealing with a population of predominantly NK1 receptors [30].

The peptide A β [Phe³¹,Nle³⁵](25–35)amide was able to displace the label completely, with an affinity approximately equal to that of neurokinin B (Figure 1), although this does not establish

Figure 3 Electron micrograph of negatively stained fibrils obtained from A β [Nle³⁵](25–35)amide

The fibrils had diameters of 5–8 nm, characteristic of amyloid fibrils. Scale bar, 100 nm.

Table 2 CD spectra of A β (25–35) and the Phe³¹ analogue

Peptide/conditions	Spectra	Interpretation
A β (25–35) in water and in buffer, pH 4.0–9.0 and A β [Phe ³¹](25–35) in water	Minimum at approx. 197 nm	Random conformation
A β (25–35) in 25%, 50%, 75% and 90% (v/v) acetonitrile	With increasing [acetonitrile], the minimum became shallower and shifted to longer wavelengths, reaching 214 nm at 90% (v/v) acetonitrile when there was also a maximum at 195 nm (see Figure 2)	A change from a random conformation to a high population of β -sheet as [acetonitrile] increases
A β [Phe ³¹](25–35) in 25%, 50%, 75% and 90% (v/v) acetonitrile	With increasing [acetonitrile], the minimum became shallower and shifted slightly to longer wavelengths, reaching 200 nm at 90% (v/v) acetonitrile	Increasing [acetonitrile] leads to a slight increase in the population of ordered conformations
A β (25–35) in 25%, 50%, 75% and 90% (v/v) TFE	With increasing [TFE], the minimum deepened and shifted slightly to longer wavelengths, reaching 200 nm with a shoulder at 226 nm. This change was complete at 75% (v/v) TFE	Increasing [TFE] leads to an increase in the population of ordered conformations, presumably including some α -helix
A β [Phe ³¹](25–35) in 25%, 50%, 75% and 90% (v/v) TFE	There was almost no change with increasing [TFE]. The minimum shifted slightly from 195 to 198 nm. There was a shallow shoulder at 226 nm at all concentrations of TFE	Increasing [TFE] leads to only a slight increase in the population of ordered conformations, which always includes some α -helix

whether the peptide would act as an agonist or an antagonist. However, A β [Nle³⁵](25–35)amide was unable to displace the label completely, even at 300 μ M. This is consistent with previously published reports [15]. Thus A β [Phe³¹,Nle³⁵](25–35)amide behaves as a typical member of the tachykinin family but A β [Nle³⁵](25–35)amide does not.

It has been reported that N-terminally extended analogues of A β (25–35)amide show enhanced binding to tachykinin receptors [16]. We tested this by studying the ability of a series of peptides {A β [Nle³⁵](25–35)amide, A β [Nle³⁵](23–35)amide, A β [Nle³⁵](21–35)amide and A β [Nle³⁵](19–35)amide} to displace the bound label. As shown in Figure 2, A β [Nle³⁵](19–35)amide did show enhanced binding compared with the shorter analogues but still

did not displace the label completely, even at 10 μ M. The peptide A β (1–40) and two natural mutants A β [Gly²¹](1–40) and A β -[Gln²²](1–40), which are associated with the occurrence of familial Alzheimer’s disease-related conditions, all failed to displace the labelled substance P at up 10 μ M peptide, as did A β (1–40) in the presence of glycine (10 μ M), glutamine (10 μ M) and CaCl₂ (1.8 mM), conditions under which A β (1–40) has been reported to activate tachykinin receptors [22] (results not shown).

Having shown that the two families of peptides behaved differently in this assay, we then began to examine their physico-chemical properties. When solutions of A β [Phe³¹,Nle³⁵](25–35)amide and A β [Nle³⁵](25–35)amide (200 μ M) were left in buffer (50 mM Tris/HCl, pH 7.4), after 4 days a precipitate was found

Table 3 CD spectra of $A\beta[Nle^{35}](25-35)$ amide and the Phe^{31} analogue

Peptide/conditions	Spectra	Interpretation
$A\beta[Nle^{35}](25-35)$ amide and $A\beta[Phe^{31},Nle^{35}](25-35)$ amide in water	Minimum at approx. 197 nm	Random conformation
$A\beta[Nle^{35}](25-35)$ amide and $A\beta[Phe^{31},Nle^{35}](25-35)$ amide in Tris/HCl buffer, pH 7.4	The Phe^{31} analogue had a minimum at 202 nm. $A\beta[Nle^{35}](25-35)$ amide had a more intense minimum at 205 nm and a maximum at 192 nm (see Figure 3)	$A\beta[Nle^{35}](25-35)$ amide has a higher population of ordered conformations in buffer at pH 7.4 than does the Phe^{31} analogue
$A\beta[Nle^{35}](25-35)$ amide in 10%, 25%, 50%, 75% and 90% (v/v) TFE	With increasing [TFE], the minimum moves from 197 to 205 nm with a shoulder at 220 nm and the spectrum becoming positive below 195 nm. The change is essentially complete at 25% TFE	Increasing [TFE] leads to an increase in the population of ordered conformations, presumably including some α -helix
$A\beta[Phe^{31},Nle^{35}](25-35)$ amide in 25%, 50%, 75% and 90% (v/v) TFE	With increasing [TFE], the minimum moves from 197 to 215 nm at 25% (v/v) TFE and then moves back to 202 nm at 50% TFE and above (see Figure 4)	With increasing [TFE], the conformation shifts from random to β -sheet and then to a significant population of α -helix

Figure 4 Effect of increasing concentrations of acetonitrile on the CD spectra of solutions of $A\beta(25-35)$ and $A\beta[Phe^{31}](25-35)$

CD spectra were obtained after the dilution of an aqueous solution of the peptide with the appropriate concentration of acetonitrile/water to give peptide (0.5 mg/ml) in the following final concentrations of acetonitrile (all v/v): solid line, $A\beta(25-35)$ 90% acetonitrile; broken line, $A\beta(25-35)$ 25% acetonitrile; dotted line, $A\beta[Phe^{31}](25-35)$ 90% acetonitrile; dot-dashed line, $A\beta[Phe^{31}](25-35)$ 25% acetonitrile.

for both peptides. The precipitates were collected and examined by electron microscopy. The precipitate from $A\beta[Nle^{35}](25-35)$ amide was found to contain characteristic amyloid fibrils of diameter 5–8 nm (Figure 3), whereas that from $A\beta[Phe^{31},Nle^{35}](25-35)$ amide did not (results not shown).

The CD spectra obtained are summarized in Tables 2 and 3. In water all peptides [$A\beta(25-35)$, $A\beta[Phe^{31}](25-35)$ and the corresponding Nle^{35} amide analogues] gave spectra with negative signals from 190 to 210 nm and a minimum at 196 nm, which is the classic spectrum expected from peptides with predominantly random conformations. Similar results were obtained for $A\beta(25-35)$ in buffer solutions over the pH range 4.0–9.0. As is common, we used organic solvents to emphasize the differing conformational tendencies of the various peptides [31]. Trifluoroethanol (TFE) was used to promote α -helical structure [31] and acetonitrile to promote β -sheet structure [32]. It is generally assumed that the solvents only promote tendencies that are in-

Figure 5 CD spectra of solutions of $A\beta[Nle^{35}](25-35)$ amide (solid line) and $A\beta[Phe^{31},Nle^{35}](25-35)$ amide (dotted line) in Tris/HCl buffer, pH 7.4

CD spectra were obtained after the dilution of an aqueous solution of the peptide with Tris/HCl buffer (final buffer concentration 50 mM) to give 0.5 mg/ml peptide.

herently present and do not induce, for example, helix formation in a peptide that has no natural ability to form a helix. As evidence of this, we have found that a peptide comprising the first 18 residues of the non- $A\beta$ -component of Alzheimer's amyloid gives a spectrum characteristic of β -sheet structure rather than α -helix when dissolved in TFE [33].

Some differences were found between all pairs of Ile^{31} and Phe^{31} peptides as described in Tables 2 and 3. Particular mention can be made of the following. (1) $A\beta(25-35)$ and $A\beta[Phe^{31}](25-35)$ in acetonitrile/water (Figure 4): with increasing acetonitrile content the spectrum of $A\beta(25-35)$ changed from one typical of a random conformation to a classic β -sheet spectrum in 90% (v/v) acetonitrile. The spectrum of $A\beta[Phe^{31}](25-35)$ also showed a change away from random structure over the same range of solvent compositions but to a much smaller extent. (2) $A\beta[Nle^{35}](25-35)$ amide and $A\beta[Phe^{31},Nle^{35}](25-35)$ amide in Tris buffer at pH 7.4 (Figure 5): the spectrum of $A\beta[Phe^{31},Nle^{35}](25-35)$ amide was essentially random, whereas that of $A\beta[Nle^{35}](25-35)$ amide showed a greater degree of order, as characterized

Figure 6 Effect of increasing concentrations of TFE on the CD spectra of solutions of $A\beta$ [Phe³¹,Nle³⁵](25–35)amide

CD spectra were obtained after the dilution of an aqueous solution of the peptide with the appropriate concentration of TFE/water to give 0.5 mg/ml peptide in the following final concentrations of TFE (all v/v): double-dot-dashed line, 95%; dot-dashed line, 50%; dotted line, 25%; broken line, 10%; solid line, 0%.

by a maximum at 192 nm. (3) $A\beta$ [Nle³⁵](25–35)amide and $A\beta$ [Phe³¹,Nle³⁵](25–35)amide in TFE/water (Figure 6): whereas the spectrum of $A\beta$ [Nle³⁵](25–35)amide showed a slight change due to an increase in the population of ordered conformations as the concentration of TFE was increased, that of $A\beta$ [Phe³¹,Nle³⁵](25–35)amide changed to a β -sheet spectrum in 25% (v/v) TFE and then to one characteristic of a more complex ordered mixture (at 50% TFE and above) that included some α -helix. Interestingly such conformationally labile behaviour has already been reported for tachykinins in membrane-mimicking environments [34].

Solution NMR spectroscopy is a technique much used in the study of peptide conformation [35]. However, amyloid peptides

are not well suited to NMR studies. NMR generally requires peptide concentrations of more than 1 mM; at this concentration many amyloid peptides undergo partial or even complete aggregation during data collection. The several groups that have used NMR spectroscopy to study $A\beta$ or $A\beta$ fragments have used various approaches to overcome this problem. For example, some have chosen to study N-terminal fragments [36,37], which have some ability to aggregate but do not deposit on existing plaques [38]. Other groups have chosen to work with conditions that prevent or at least minimize aggregation, e.g. DMSO [39], 40% (v/v) aqueous TFE [40] or lithium dodecyl sulphate [41]. Only Maggio and co-workers [38,42] have chosen to work in aqueous solution with $A\beta$ (10–35)amide at pH 5.6; the peptide deposits on plaques at approx. 37% of the rate of the full-length peptide. They achieved their results by working with a more dilute solution (approx. 250 μ M) but using a larger than usual volume of solution (10 mm NMR tube) and the most sensitive instrumentation available (750 MHz). Those working in membrane-mimicking environments (TFE or SDS) have generally found some α -helix, especially for the N-terminal residues, but Maggio and co-workers found no evidence for this under conditions in which $A\beta$ (10–35)amide is competent to add to plaques; they interpret their results in terms of a folded structure. Two pieces of evidence led them to this conclusion. First, the existence of some very low $^3J_{NH\alpha}$ values for residues (e.g. Glu¹¹, Gln¹⁵, Lys¹⁶, Ala²¹ and Glu²²) is strong evidence because such low values rarely occur except where a single, folded conformation predominates; secondly, many more NOE cross-peaks occurred in NOESY spectra from the plaque-competent peptide $A\beta$ (10–35)amide than were found for peptides incapable of forming plaques, i.e. $A\beta$ (1–28) or $A\beta$ [Thr¹⁹](10–35)amide. However, the increased number of NOEs observed might arise not simply from a folded structure but also because the plaque-competent $A\beta$ (10–35)amide (but not the other peptides) exists as a dimer or other oligomer with a higher 'molecular mass' and would therefore be expected to have a longer correlation time with enhanced NOE relative to the monomer. Recently several groups [43–45] have reported that $A\beta$ (1–40) exists in aqueous solution as a dimer or higher oligomer, even at nanomolar concentrations.

Figure 7 One-dimensional 1H NMR spectra of $A\beta$ (25–35) and $A\beta$ [Phe³¹](25–35) in $[^2H_6]$ DMSO

Table 4 Chemical shift assignments for A β (25–35)

The chemical shifts are quoted relative to trimethylsilane.

Residue	Chemical shift (p.p.m.)				
	NH	α -CH	β -CH	γ -CH	Other
Gly		3.70			
Ser	8.59	4.47	3.58/3.52		
Asn	8.43	4.57	2.60/2.49		γ -NH ₂ 7.47/7.02
Lys	8.00	4.16	1.68/1.50	1.30/1.30	δ -CH ₂ 1.52; ϵ -CH ₂ 2.75
Gly	8.16	3.75/3.65			
Ala	7.90	4.33	1.18		
Ile	7.96	4.16	1.72	1.42/1.07	γ -CH ₃ 0.81; δ -CH ₃ 0.79
Ile	7.77	4.15	1.72	1.42/1.07	γ -CH ₃ 0.81; δ -CH ₃ 0.79
Gly	8.13	3.75/3.65			
Leu	7.92	4.34	1.50/1.42	1.60	δ -CH ₃ 0.87/0.84
Met	8.18	4.25	1.95/1.86	2.46	ϵ -CH ₃ 2.03

DMSO was used as solvent in our studies because it seems to prevent the aggregation and precipitation of A β peptides [44,46], which we have observed to occur in both aqueous solutions and in mixed aqueous/acetonitrile solutions at lower concentrations than those used for these NMR studies. Against this pragmatic choice of DMSO, it might be argued that DMSO is not very relevant to intracellular or extracellular conditions. In defence of our choice, we note that Sorimachi and Craik [47] have assembled considerable evidence that DMSO does not necessarily abolish either biological activity or conformational preferences in peptides. Further, Amodeo et al. [48] have noted that the liquid phases in the cytoplasm, in the intersynaptic space and within receptor-binding sites are characterized by a higher viscosity than bulk water. They suggest that DMSO (viscosity approx. 2.5 times that of water) or other high-viscosity liquids might be more realistic than water in modelling these environments. It was also felt that DMSO provides a more relevant environment with respect to aggregation than membrane-mimicking conditions, e.g. lithium dodecyl sulphate [41] or aqueous TFE [40], which tend to promote α -helix formation. Recent studies do indicate interactions between the A β peptide and membranes or membrane components [49]. However, these interactions seem to be between the peptide and the charged heads of the membrane lipids and, unlike the situation with dodecyl sulphate salts or

Table 5 Chemical shift assignments for A β [Phe³¹](25–35)

The chemical shifts are quoted relative to trimethylsilane.

Residue/proton	Chemical shift (p.p.m.)				
	NH	α -CH	β -CH	γ -CH	Other
Gly		3.70			
Ser	8.58	4.42	3.58		
Asn	8.40	4.58	2.58/2.47		γ -NH ₂ 7.44/6.99
Lys	7.98	4.18	1.50	1.32	δ -CH ₂ 1.53; ϵ -CH ₂ 2.55
Gly	8.12	3.65			
Ala	7.90	4.25	1.14		
Phe	8.10	4.52	3.04/2.80		Ar 7.23/7.17
Ile	7.80	4.18	1.70	1.42/1.07	γ -CH ₃ 0.87; δ -CH ₃ 0.80
Gly	8.14	3.74			
Leu	7.90	4.35	1.43	1.60	δ -CH ₃ 0.89/0.84
Met	8.22	4.28	1.98/1.88	2.48	δ -CH ₃ 2.03

Table 6 Temperature coefficients of amide proton chemical shifts in A β (25–35) and A β [Phe³¹](25–35)

A dash signifies that the peak was obscured by other resonances.

Residue	10 ³ × Temperature coefficient (p.p.m./K)	
	A β (25–35)	A β [Phe ³¹](25–35)
Gly		
Ser	−3.80	−5.28
Asn	−4.84	−7.08
Asn CONH ₂	−3.31/−4.44	−5.06/−6.77
Lys	−3.76	−4.70
Gly	−3.77	—
Ala	−4.45	−6.12 or −7.28
Ile/Phe	−3.27	—
Ile	−4.34	−7.04
Gly	−4.41	—
Leu	−4.25	−7.28 or −6.12
Met	−5.22	−8.35

	Gly	Ser	Asn	Lys	Gly	Ala	Ile	Ile	Gly	Leu	Met
³ J _{Nα}	8.0	7.5	7.5		8.0	9.0	8.0		8.0		
d _{NN}											
d _{αN}											
	Gly	Ser	Asn	Lys	Gly	Ala	Phe	Ile	Gly	Leu	Met
³ J _{Nα}	7.7	7.7			7.7		8.2		7.7	7.7	
d _{NN}											
d _{αN}											

Figure 8 ¹H coupling constants (Hz) and d_{NN} and d _{α N} connectivities observed for A β (25–35) and A β [Phe³¹](25–35) in [²H₆]DMSO solution

TFE, also seem to promote the β -sheet structure that is essential for aggregation and neurotoxicity.

One-dimensional ¹H NMR spectra of both peptides in DMSO solution are shown in Figure 7. The detailed assignments of resonances for both peptides are given in Tables 4 and 5. Temperature coefficients for both A β (25–35) and A β [Phe³¹](25–35) are given in Table 6. The major NOE connectivities observed for both peptides are shown in Figure 8. In general the assignments made for both peptides lie close to the values reported for the corresponding amino acid residue in peptides with random conformations [50]. This is not unexpected because DMSO has the reputation of strongly solvating peptides and ‘destroying’ structure. Our hope was that some structural differences would persist between the peptides even in DMSO solution.

There was a better dispersion of the amide chemical shift values for A β (25–35) than for A β [Phe³¹](25–35). For the former peptide all the amide resonances could be distinguished at 25 °C (and at least at three or four other temperatures between 25 and 50 °C). For A β [Phe³¹](25–35) some amide resonances were obscured by overlapping at 25 °C and at all other temperatures

examined. It is of some interest that the amide chemical shifts for the two Ile residues in A β (25–35) were significantly different, implying that the structure of this peptide is not totally random.

For peptides dissolved in DMSO, values of the amide temperature coefficient greater than -3 p.p.b./K are usually taken to indicate fairly extensive shielding from exposure to solvent, whereas values less than -5 p.p.b./K are usually taken as indicating full exposure to solvent [51]. The values that could be determined for A β [Phe³¹](25–35) are all less than -5 p.p.b./K, indicating that in this peptide these amide protons are fully exposed to solvent, i.e. the peptide does not aggregate or adopt a folded conformation to any significant extent. For A β (25–35) all the amide temperature coefficients lie between -3 and -5 p.p.b./K. Values in this range might indicate some shielding from solvent but are not usually judged very significant. In this case however, these values can be deemed significant by comparison with those of A β [Phe³¹](25–35). Quite clearly in comparison with those of A β [Phe³¹](25–35), the amide protons of A β (25–35) are significantly shielded from solvent, presumably by involvement in intermolecular or intramolecular hydrogen-bonding.

For both peptides, most of the NOE connectivities observed were between protons on neighbouring residues (see Figure 6), which does not yield much information about conformation. Essentially the same connectivities were present in both NOESY and ROESY spectra. For both peptides a complete set of $d_{\alpha\alpha}$ and $d_{\alpha N}$ connectivities was observed linking the α - and amide protons of residues from Ser²⁶ to Met³⁵ and the intensities of the $d_{\alpha N}$ connectivities were uniformly greater than those of the $d_{\alpha\alpha}$ connectivities. This would indicate a significant contribution from extended conformations [52]. In contrast, for neither peptide was a complete set of d_{NN} connectivities observed. These would indicate contributions from folded conformations [52]. As can be seen from Figure 6, a more extensive set of more intense d_{NN} connectivities was observed for A β (25–35), indicating that the conformational average for A β [Phe³¹](25–35) might be more rigidly extended.

For A β (25–35), but not A β [Phe³¹](25–35), two NOE connectivities between non-neighbouring residues were also observed. A fairly intense cross-peak between the α -proton of Ser²⁶ and the amide proton of Met³⁵ was observed at all mixing times, whereas a weaker cross-peak between the α -proton of Asn²⁷ and the amide proton of Gly²⁹ was observed in a ROESY spectrum with a mixing time of 700 ms. Because the latter, which is consistent with some kind of bend involving residues Asn²⁷ to Gly²⁹, was observed only at long mixing times, the conformation from which it arises obviously makes only a minor contribution to the total ensemble of conformations present. The former connectivity is consistent with either a linear anti-parallel β -sheet, which implies at least the formation of a dimer, or with a β -hairpin structure, which could be present in either associated or unassociated molecules.

Thus both temperature studies and NOE measurements show differences between the two peptides. Both techniques provide evidence that A β [Phe³¹](25–35) exists as unassociated molecules in random (mainly extended) conformation, whereas A β (25–35) exhibits partial association and/or a partly folded structure. The temperature coefficients for A β (25–35) vary slightly between residues but not in a regular pattern that could be correlated with a simple β -sheet either between molecules or within a β -hairpin, so that some more complex averaging of conformations must be postulated. Such conclusions do not contradict previous findings ([44] and references therein) that A β (1–40) is effectively monomeric in DMSO. It has been established that C-terminal residues are much more important for association than N-terminal

residues and it might be expected that peptides lacking N-terminal residues show stronger association than those possessing them [53]. Thus it is not unreasonable that A β (1–40) should be almost monomeric in DMSO, whereas A β (25–35) might be significantly associated. As a somewhat extreme example of this type of behaviour, we can report that in electrospray ionization mass spectra of A β (31–35) and A β (32–35), peaks due to dimers (m/z 1092 and 865 respectively) as well as monomers (m/z 546 and 433 respectively) were present. These spectra were obtained from solutions of the peptides in water/acetonitrile/formic acid, which would be expected to disrupt all but the tightest associations. This might have some relevance to the recent suggestion that amyloid neurotoxicity might be mediated by soluble dimers and oligomers of A β peptides as well as (or rather than) by insoluble aggregates [43].

In conclusion, with all of the techniques used some differences have been detected between the A β (25–35) peptides and their Phe³¹ analogues. In particular, A β [Phe³¹](25–35)amide displays some typical tachykinin behaviour, whereas A β (25–35)amide does not. These differences should perhaps not be entirely unexpected. As referred to above, tachykinins are characterized by an aromatic residue at the fifth position from the C-terminus, whereas Ile³¹ has been shown to be important for the full expression of the physicochemical and biological properties of amyloid peptides [54,55]. It seems reasonable to infer that if A β (25–35) and A β (25–35)amide sequences share any activities with tachykinins, they are not mediated via binding to the classical tachykinin receptors. It would be interesting to extend these studies by examining both peptides in solution and as solids, perhaps with Fourier-transform infrared methods, which are applicable to both states.

We thank Dr. J. A. Ballantine and his staff (EPSRC National Mass Spectrometry Service Centre at the University of Wales, Swansea, Glamorgan, Wales, U.K.), for performing and assisting in the interpretation of electrospray ionization MS; Mr. R. Murphy (School of Chemistry, The Queen's University of Belfast, Belfast, N. Ireland, U.K.) for assistance with CD studies; and Ms. O. O'Shea and Mr. P. Larkin (School of Biomedical Science, The Queen's University of Belfast, Belfast, N. Ireland, U.K.) for assistance with electron microscopy. The support of the Libyan government for the work of O.M.A.E.-A. is acknowledged.

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